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ORIGINAL ARTICLE

Two distinct mutations in *gyrA* lead to ciprofloxacin and nalidixic acid resistance in *Campylobacter coli* and *Campylobacter jejuni* isolated from chickens and beef cattle*

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Abstract

Aims: The aim of this study was to identify point mutations in the *gyrA* quinolone resistance determining region (QRDR) of *Campylobacter coli* ($n = 27$) and *Campylobacter jejuni* ($n = 26$) that confer nalidixic acid (NAL) resistance without conferring resistance to ciprofloxacin (CIP).

Methods and Results: Point mutations in the QRDR of *gyrA* from *C. coli* and *C. jejuni* isolates were identified by direct sequencing. All isolates ($n = 14$) with minimum inhibitory concentrations (MICs) ≥ 4 μg ml^{-1} for CIP and ≥ 32 μg ml^{-1} for NAL possessed a missense mutation leading to substitution of Ile for Thr at codon 86. Three isolates with a missense mutation leading to a Thr86Ala substitution had MICs <4 μg ml^{-1} for CIP and ≥ 32 μg ml^{-1} for NAL.

Conclusions: These data confirm previous findings that Thr86Ile mutations confer resistance to both CIP and NAL. However, resistance to NAL alone was conferred by a single Thr86Ala mutation.

Significance and Impact of the Study: Resistance to NAL alone arises independently from CIP resistance. In addition, the role of other previously described point mutations in quinolone resistance is discussed.

Introduction

Campylobacter is recognized as a major cause of acute bacterial gastroenteritis in humans worldwide (Friedman *et al.* 2000). *Campylobacter coli* and *Campylobacter jejuni* are commonly associated with human illness and produce similar symptoms. Clinical signs include abdominal pain, fever, malaise, nausea, vomiting and diarrhoea (Skirrow and Blaser 2000). Most patients recover in less than a week, but up to 20% may relapse or experience prolonged or severe illness requiring antibiotic therapy. Fluoroquinolones are commonly used for human treatment of campylobacteriosis, and as such, resistance to fluoroquinolones is an emerging public health concern (Skirrow and Blaser 2000).

Quinolones and fluoroquinolones directly inhibit DNA synthesis by interfering with DNA gyrase, a type II topoisomerase (Maxwell 1992). In *C. coli* and *C. jejuni*, resistance to the quinolone nalidixic acid (NAL) and the fluoroquinolone ciprofloxacin (CIP) is most commonly attributed to a single base mutation of the *gyrA* gene at codon 86, which produces an amino acid substitution of isoleucine for threonine (Zirnstein *et al.* 1999,2000; Niwa *et al.* 2003; Padungtod *et al.* 2003; Chuma *et al.* 2004; Payot *et al.* 2004). In addition to isoleucine, alanine (Beckmann *et al.* 2004; Chu *et al.* 2004; McIver *et al.* 2004) and lysine (Luo *et al.* 2003; Chu *et al.* 2004) have also been reported as amino acid substitutions for threonine and may be associated with resistance to CIP and NAL in *Campylobacter*. Additionally, point mutations in *gyrA* that

produce amino acid substitutions at codon 70 (Ala → Thr) (Wang *et al.* 1993), codon 90 (Asp → Asn) (Wang *et al.* 1993; Hakanen *et al.* 2002; Luo *et al.* 2003; Beckmann *et al.* 2004; McIver *et al.* 2004) and codon 104 (Pro → Ser) (Zirnstein *et al.* 1999; Hakanen *et al.* 2002; Piddock *et al.* 2003; Beckmann *et al.* 2004) have been observed. Ge *et al.* (2003) have also reported amino acid substitutions at codon 22 (Ser → Gly), codon 203 (Asn → Ser) and codon 206 (Ala → Thr) caused by point mutations in *gyrA*.

This study was conducted to examine the sequences of *gyrA* from *C. coli* and *C. jejuni* isolates with varying levels of resistance to CIP and NAL. PCR primers were designed that can be used to amplify and sequence *gyrA* from both *C. coli* and *C. jejuni*, including the region of *gyrA* upstream of the quinolone resistance determining region (QRDR). To date, the *C. coli* sequences submitted to GenBank have not included the *gyrA* start codon (accession numbers AF092101, AJ491808 and AY575048), and this portion of the gene is included in the amplicon.

Materials and methods

Bacterial strains and culture conditions

A total of 27 *C. coli* isolates and 26 *C. jejuni* isolates from chicken carcass rinses and beef cattle faeces were selected from the *Campylobacter* collection at the USDA-ARS Russell Research Center in Athens, GA. Isolates were chosen for resistance to both CIP and NAL, resistance to NAL only, or resistance to neither CIP nor NAL, determined as described below. The isolates were diverse in origin and epidemiologically unrelated: strains were isolated in 1999, 2000 or 2002 from samples collected in 17 US states. *Campylobacter* isolates were routinely stored as frozen stocks at −80°C in Mueller-Hinton broth supplemented with 10% glycerol. *Campylobacter* isolates were recovered from frozen stocks on Campy-Cefex agar plates (Stern *et al.* 1992) and incubated under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) in zip-top bags for 48 h at 42°C. Single isolates were plated onto fresh Campy-Cefex plates, incubated as described previously, and used for species confirmation and antimicrobial resistance assays. Speciation of *Campylobacter* isolates was performed using the *Campylobacter* BAX® PCR (DuPont™ Qualicon, Wilmington, DE, USA) as previously described (Englen and Fedorka-Cray 2002).

Antimicrobial susceptibility testing

Isolates were tested for antimicrobial susceptibility using the E test (AB-Biodisk, Piscataway, NJ, USA) according to manufacturer's directions. The E test method has been shown to give results comparable to other methods such as agar dilution for *Campylobacter* (Luber *et al.* 2003; Oncul *et al.* 2003). In brief, 150 mm Mueller-Hinton + 5% lysed horse blood plates (BD Diagnostic Systems, Sparks, MD, USA) were inoculated with 100 μl of a cell suspension equivalent to a 1·0 McFarland standard. The inoculum was swabbed evenly across the entire plate surface. E test strips were brought to room temperature from −20°C storage before use. Two strips were laid at 180° angles onto each plate. The plates were placed in zip-top bags and incubated microaerobically for 48 h at 42°C as described previously. Following incubation, the point at which the zone of growth inhibition intersected the strip was read as the minimum inhibitory concentration (MIC) of the antimicrobial in $\mu g\ ml^{-1}$. The MICs for the respective resistant break points were those as reported in the CDC NARMS 2001 Annual Report (http://www.cdc.gov/narms/annuals.htm): CIP, $\geq 4\ \mu g\ ml^{-1}$ and NAL, $\geq 32\ \mu g\ ml^{-1}$. Colonies of *C. coli* and *C. jejuni* were collected from the E test plates and used for genomic DNA isolation.

DNA isolation and gyrA PCR conditions

Genomic DNA was isolated from each of the *C. coli* and *C. jejuni* isolates with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to manufacturer's directions. The *C. jejuni gyrA* GenBank sequence L04566 was analysed to design PCR primers for the amplification of a region of *gyrA* that included the QRDR. The PCR primers gyrAF, 5′-GCT CTT GTT TTA GCT TGA TGC A-3′, and gyrAR, 5′-TTG TCG CCA TC CTA CAG CTA-3′, generate a 620 bp PCR product, including the 210 bp *gyrA* QRDR (Wang *et al.* 1993) from *C. jejuni* and *C. coli*. The PCR reactions contained 1 μl prepared genomic DNA (∼200 ng), 5 μl 10× PCR buffer, 2 μl 10 mM dNTPs, 50 pmol each primer, 0·5 μl JumpStart Taq polymerase (Sigma, St. Louis, MO, USA) and water to a final volume of 50 μl. PCRs were carried out on an Applied Biosystems GeneAmp PCR Sytem 9700 under the following conditions: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 7 min. Five microlitre samples from each PCR were analyzed by horizontal gel electrophoresis followed by staining with ethidium bromide.

DNA sequencing and sequence analysis

The remainder of each PCR reaction for *gyrA* was purified using the QIAquick PCR purification system (Qiagen, Valencia, CA, USA) for use in sequencing reactions. Sequencing reactions were carried out using the BigDye Terminator 1·1 Cycle Sequencing Kit (Applied Biosystems,

Foster City, CA, USA) according to manufacturer's directions. Duplicate forward and reverse sequencing reactions were run with primers gyrAF and gyrAR, respectively, for each isolate. Labelled sequencing reactions were purified via ethanol precipitation and run on an ABI Prism 3100 Genetic Analyzer. DNA sequences were analyzed with Vector NTI Suite (Invitrogen, Carlsbad, CA, USA). Consensus sequences were determined for *C. coli* and *C. jejuni gyrA*, and observed *gyrA* base pair changes were assigned based on deviation from consensus.

Results

Resistance to ciprofloxacin and nalidixic acid

The isolates used in this study and their respective MICs for CIP and NAL are shown in Table 1. Of the 53 isolates tested, eleven isolates (MTL03, MTL05, MTL06, MTL09, MTL18, MTL31, MTL32, MTL35, MTL38, MTL41 and MTL49) had MICs <4 μg ml^{-1} for CIP and ≥32 μg ml^{-1} for NAL. Fourteen isolates (MTL07, MTL08, MTL14, MTL15, MTL21, MTL23, MTL27, MTL30, MTL34, MTL37, MTL40, MTL42, MTL45 and MTL52) had MICs ≥4 μg ml^{-1} for CIP and ≥32 μg ml^{-1} for NAL. The remaining 28 isolates had MICs <4 μg ml^{-1} for CIP and <32 μg ml^{-1} for NAL. No isolates resistant solely to CIP were observed.

Sequence of gyrA from C. coli and C. jejuni isolates

An alignment of the deduced amino acid consensus sequences of *C. coli* and *C. jejuni* GyrA from this study with published sequences are shown in Fig. 1. The observed *C. coli* and *C. jejuni* GyrA deduced amino acid consensus sequences share 86% similarity. The deduced *C. coli* GyrA sequence shares 86, 89 and 100% similarity with GyrA from *C. jejuni* NCTC 11168, *C. fetus* U25640 and *C. coli* RM2228, respectively, while the deduced *C. jejuni* GyrA sequence shares 99, 87 and 85% similarity with GyrA from *C. jejuni* NCTC 11168, *C. fetus* U25640 and *C. coli* RM2228, respectively.

The observed sequences of *gyrA* codons Ser22 and Thr86 with their respective amino acids in the GyrA QRDRs for all isolates are shown in Table 1. A Thr86Ile (ACT → ATT) missense mutation was observed in sequences from *C. coli* isolates MTL07, MTL08, MTL14, MTL15, MTL21, MTL23 and MTL27. A Thr86Ile (ACA → ATA) missense mutation was observed in sequences from *C. jejuni* isolates MTL30, MTL34, MTL37, MTL40, MTL42, MTL45 and MTL52. A Thr86Ala (ACA → GCA) missense mutation was observed in sequences from *C. jejuni* strains MTL35, MTL38 and MTL41. In addition, a Ser22Gly (AGT → GGT) missense

Table 1 *Campylobacter* isolates used in this study, CIP and NAL MICs, and sequences of *gyrA* codons 22 and 86

			MIC μg ml^{-1}		AA (codon sequence)	
Isolate	Species	Source	CIP	NAL	Ser22	Thr86
MTL25	*C. coli*	Chicken	0·047	1·5	S(AGT)	T(ACT)
MTL17	*C. coli*	Chicken	0·047	24	S(AGT)	T(ACT)
MTL26	*C. coli*	Cattle	0·064	2	S(AGT)	T(ACT)
MTL18	*C. coli*	Chicken	0·064	256	S(AGT)	T(ACT)
MTL01	*C. coli*	Chicken	0·094	12	S(AGT)	T(ACT)
MTL24	*C. coli*	Chicken	0·094	12	S(AGT)	T(ACT)
MTL05	*C. coli*	Chicken	0·094	128	S(AGT)	T(ACT)
MTL20	*C. coli*	Chicken	0·125	6	S(AGT)	T(ACT)
MTL11	*C. coli*	Chicken	0·125	8	S(AGT)	T(ACT)
MTL02	*C. coli*	Chicken	0·125	12	S(AGT)	T(ACT)
MTL13	*C. coli*	Chicken	0·125	16	S(AGT)	T(ACT)
MTL09	*C. coli*	Chicken	0·125	64	S(AGT)	T(ACT)
MTL06	*C. coli*	Chicken	0·125	256	S(AGT)	T(ACT)
MTL10	*C. coli*	Chicken	0·19	4	S(AGT)	T(ACT)
MTL22	*C. coli*	Chicken	0·19	6	S(AGT)	T(ACT)
MTL12	*C. coli*	Chicken	0·19	8	S(AGT)	T(ACT)
MTL16	*C. coli*	Chicken	0·19	16	S(AGT)	T(ACT)
MTL04	*C. coli*	Chicken	0·19	24	S(AGT)	T(ACT)
MTL19	*C. coli*	Chicken	0·25	6	S(AGT)	T(ACT)
MTL03	*C. coli*	Chicken	0·5	48	S(AGT)	T(ACT)
MTL07	*C. coli*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL08	*C. coli*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL14	*C. coli*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL15	*C. coli*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL21	*C. coli*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL23	*C. coli*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL27	*C. coli*	Cattle	32	256	S(AGT)	**I**(A**T**T)
MTL29	*C. jejuni*	Chicken	0·023	1	S(AGT)	T(ACA)
MTL43	*C. jejuni*	Chicken	0·047	1.5	S(AGT)	T(ACA)
MTL44	*C. jejuni*	Chicken	0·047	1.5	S(AGT)	T(ACA)
MTL36	*C. jejuni*	Chicken	0·047	8	S(AGT)	T(ACA)
MTL39	*C. jejuni*	Chicken	0·047	8	S(AGT)	T(ACA)
MTL41	*C. jejuni*	Chicken	0·094	256	S(AGT)	**A**(**G**CA)
MTL47	*C. jejuni*	Cattle	0·125	6	S(AGT)	T(ACA)
MTL53	*C. jejuni*	Cattle	0·125	8	S(AGT)	T(ACA)
MTL50	*C. jejuni*	Cattle	0·125	12	S(AGT)	T(ACA)
MTL33	*C. jejuni*	Chicken	0·147	3	S(AGT)	T(ACA)
MTL48	*C. jejuni*	Cattle	0·19	4	S(AGT)	T(ACA)
MTL32	*C. jejuni*	Chicken	0·19	64	S(AGT)	T(ACA)
MTL31	*C. jejuni*	Chicken	0·19	256	S(AGT)	T(ACA)
MTL35	*C. jejuni*	Chicken	0·19	256	**G**(**G**GT)	**A**(**G**CA)
MTL28	*C. jejuni*	Chicken	0·25	6	S(AGT)	T(ACA)
MTL46	*C. jejuni*	Cattle	0·25	16	S(AGT)	T(ACA)
MTL51	*C. jejuni*	Cattle	0·32	2	S(AGT)	T(ACA)
MTL49	*C. jejuni*	Cattle	0·38	32	S(AGT)	T(ACA)
MTL38	*C. jejuni*	Chicken	0·75	256	S(AGT)	**A**(**G**CA)
MTL30	*C. jejuni*	Chicken	32	256	**G**(**G**GT)	**I**(A**T**T)
MTL34	*C. jejuni*	Chicken	32	256	**G**(**G**GT)	**I**(A**T**T)
MTL37	*C. jejuni*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL40	*C. jejuni*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL42	*C. jejuni*	Chicken	32	256	S(AGT)	**I**(A**T**T)
MTL45	*C. jejuni*	Chicken	32	256	**G**(**G**GT)	**I**(A**T**T)
MTL52	*C. jejuni*	Cattle	32	256	S(AGT)	**I**(A**T**T)

CIP, ciprofloxacin; NAL, nalidixic acid. Observed mutations are shown in bold.

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C.coli          -MENIFNKDSDIELIDIENSIKSSYLDYSMSVIIGRALPDARDGLKPVHR      NDLG
RM2228          -MENIFNKDSDIELIDIENSIKSSYLDYSMSVIIGRALPDARDGLKPVHR      NDLG
U25640          MEENIFSSNQDIDAIDVEDSI
C.jejuni        -MENIFSKDSDIELVDIENSIKSSYLDYSMSVIIGRALPDARDGLKPVHR      QNDE
11168           -MENIFSKDSDIELVDIENSIKSSYLDYSMSVIIGRALPDARDGLKPVHR      QNDE
S.enterica      ----MSDLAREITPVNIEEELKSSYLDYAMSV
E.coli          ----MSDLAREITPVNIEEELKSSYLDYAM
                         *     *
                                      ▼
C.coli          VGSRSAYKKSARIVGDVIGKYHPHGDTAVYDALVRMAQDFSMRYPSIDGQGNFGSIDGDG
RM2228          VGSRSAYKKSARIVGDVIGKYHPHGDTAVYDALVRMAQDFSMRYPSIDGQGNFGSIDGDG
U25640          VGSRSPYKKSARIVGDVIGKY
C.jejuni        AKSRTDFVKSARIVGAVIGRYHPHGDTAVYDALVRMAQDFSMRYPSITGQGNFGSIDGDS
11168           AKSRTDFVKSARIVGAVIGRYHPHGDTAVYDALVRMAQDFSMRYPSITGQGNFGSIDGDS
S.enterica      NDWNKAYKKSARVVGDVIGKYHPHGDSAVYDT
E.coli          NDWNKAYKKSARVVGDVIGKYHPHGDSAVY
                       **** ** *** *
                                              ▲
C.coli          AAAMRYTEARMTILAEELLRDIDKDTVDFVPNYDDSMSEPDVLPARVPNLLLNGSSGIAV
RM2228          AAAMRYTEARMTILAEELLRDIDKDTVDFVPNYDDSMSEPDVLPARVPNLLLNGSSGIAV
U25640          AAAMRYTEARMTVLAEELLRD
C.jejuni        AAAMRYTEAKMSKLSHELLKDIDKDTVDFVPNYDGSESEPDVLPSRVPNLLLNGSSGIAV
11168           AAAMRYTEAKMSKLSHELLKDIDKDTVDFVPNYDGSESEPDVLPSRVPNLLLNGSSGIAV
S.enterica      AAAMRYTEIRLAKIAHELMADLEKETVDFVDN
E.coli          AAAMRYTEIRLAKIAHELMADLEKETVDFV
                ********        **  *

C.coli          GMAT
RM2228          GMAT
U25640          GMAT
C.jejuni        GMAT
11168           GMAT
S.enterica      GMAT
E.coli          GMAT
                ****
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Figure 1 Alignment of *C. coli* and *C. jejuni* GyrA deduced amino acid consensus sequences from this study, with published GyrA sequences. *C. coli*, consensus *C. coli* amino acid sequence from this study; *C. jejuni*, consensus *C. jejuni* amino acid sequence from this study; RM2228, *C. coli* RM2228, accession number NZ_AAFL00000000; 11168, *C. jejuni* NCTC11168, accession number AL139077; U25640, *Campylobacter fetus*, accession number U25640; *S. enterica*, *Salmonella enterica* Typhimurium LT2, accession number AE008801; *E. coli*, *Escherichia coli* CFT073, accession number AE016763. *, 100% similarity; ▼, Ser83/Thr86; ▲, Asp87/Asp90. Similar amino acids found in *C. coli* and *C. jejuni* are in bold.

mutation was observed in sequences from *C. jejuni* isolates MTL30, MTL34, MTL35 and MTL45.

The observed silent mutations from this study are shown in Table 2. A total of 26 *C. jejuni* isolates possessed one or more silent mutations, while 12 *C. coli* isolates possessed one or more silent mutations. One isolate, *C. jejuni* MTL44, was found to possess eight silent mutations. In *C. coli* isolates MTL05, MTL06, MTL26 and MTL27, a silent mutation at Phe99 (TTC → TTT) was observed. It should be noted that the Phe99 codon TTT is found in *C. jejuni*.

Amino acid sequence of gyrA and resistance to ciprofloxacin and nalidixic acid

The deduced amino acid sequences were compared to the MICs of CIP and NAL for each of the isolates (Table 1). All *C. coli* and *C. jejuni* isolates with MICs ≥4 μg ml^{-1} for CIP and ≥32 μg ml^{-1} for NAL had the Thr86Ile substitution. *C. jejuni* MTL35, MTL38 and MTL41 had a Thr86Ala substitution and had MICs <4 μg ml^{-1} for CIP and ≥32 μg ml^{-1} for NAL. Interestingly, eight isolates (MTL03, MTL05, MTL06, MTL09, MTL18, MTL31, MTL32 and MTL49) had MICs ≥32 μg ml^{-1} for NAL, but no detectable *gyrA* mutations. The Ser22Gly substitutions appeared to have no affect on quinolone resistance, as each of these isolates also possessed either the Thr86Ile (MTL30, MTL34 and MTL45) or Thr86Ala (MTL35) substitution, which accounted for the observed CIP and NAL MICs.

Discussion

A number of studies have examined the mechanisms of quinolone resistance in *Campylobacter*, with the majority

Table 2 Silent mutations detected in this study. Mutations are based on deviation from consensus sequence for *C. jejuni* or *C. coli*

No. of Isolates	Species	Source	Codon/Consensus sequence 39 GAC	67 GTC	78 GGT	81 CAC	82 CCA	92 TTG	99* TTT/TTC	110 GGC	117 GGT	119 AGC	120 GCC	136 GAG	157 AGT	161 GTT	166 GTG	22 AGT	86† ACA/ACT
1	*C. jejuni*	Chicken	GAT	GTT			CCG	TTA		GGT	GGC	AGT	GCT						
1	*C. jejuni*	Cattle			GGC														
4	*C. jejuni*	Chicken				CAT							GCT			GTC			
2	*C. jejuni*	Chicken				CAT							GCT			GTC			**G**CA
1	*C. jejuni*	Chicken				CAT							GCT			GTC		**G**GT	**G**CA
3	*C. jejuni*	Chicken				CAT							GCT			GTC		**G**GT	ATA
3	*C. jejuni*	Chicken				CAT							GCT			GTC			ATA
7	*C. jejuni*	Cattle										AGT			AGC				
3	*C. jejuni*	Cattle												GAA					
1	*C. jejuni*	Cattle												GAA					ATA
3	*C. coli*	Chicken	GAT												AGC		GTA		
3	*C. coli*	Chicken	GAT						TTT										
4	*C. coli*	Chicken	GAT																
1	*C. coli*	Cattle	GAT						TTT										ATT
1	*C. coli*	Chicken	GAT												AGC		GTA		ATT
5	*C. coli*	Chicken																	ATT

*TTT is used by *C. jejuni* at codon 99, TTC is used by *C. coli* at codon 99. †ACA is used by *C. jejuni* at codon 86, ACT is used by *C. coli* at codon 86. Missense mutations are shown in bold.

focused on point mutations in *gyrA* that lead to resistance to these antibiotics (Zirnstein *et al.* 1999,2000). As in previous reports, we found that the Thr86Ile GyrA substitution was the most common mutation among *C. coli* and *C. jejuni* isolates resistant to both CIP and NAL. However, previous studies have failed to distinguish *gyrA* mutations that confer only NAL resistance and those that also confer cross-resistance to CIP. For example, several studies identified *gyrA* mutations that were associated with CIP resistance in *Campylobacter*, but provided no data on NAL resistance (Ge *et al.* 2003; Piddock *et al.* 2003; Dionisi *et al.* 2004). Other reports have focused specifically on detection of the Thr86Ile *gyrA* mutation (Wilson *et al.* 2000; Zirnstein *et al.* 2000). In order to differentiate the mechanisms of resistance to NAL alone from those that confer resistance to CIP, we examined isolates that were only resistant to NAL. In three isolates, a Thr86Ala GyrA substitution rather than the Thr86Ile substitution was found. The role of the Thr86Ala GyrA substitution in NAL resistance has been described previously (Bachoual *et al.* 2001; Beckmann *et al.* 2004; Chu *et al.* 2004; McIver *et al.* 2004) and our results demonstrate that this *gyrA* mutation confers NAL resistance, but not CIP resistance.

Although *gyrA* mutations leading to Asp90 substitutions were not detected in our study, Asp90 GyrA substitutions have been identified in quinolone resistant *Campylobacter* in previous studies (Wang *et al.* 1993; Hakanen *et al.* 2002; Luo *et al.* 2003; Piddock *et al.* 2003; Beckmann *et al.* 2004; McIver *et al.* 2004). Just as codon Thr86 in *C. jejuni* GyrA is equivalent to codon Ser83 in *Escherichia coli* GyrA (Wang *et al.* 1993; Willmott and Maxwell 1993), Asp90 in *Campylobacter* is equivalent to Asp87 in *E. coli* (Wang *et al.* 1993) and *Salmonella enterica* Typhimurium LT2 (Fig. 1). Results from studies of quinolone resistance in *S. enterica* (Hirose *et al.* 2002; Ling *et al.* 2003) support previously described evidence for the role of Asp90 GyrA substitutions in *Campylobacter* quinolone resistance (Wang *et al.* 1993; Luo *et al.* 2003; Beckmann *et al.* 2004; McIver *et al.* 2004).

The contribution of other reported mutations in *gyrA* to CIP and NAL resistance is unclear, as multiple mutations have often been reported together (Zirnstein *et al.* 1999; Hakanen *et al.* 2002; Ge *et al.* 2003; Beckmann *et al.* 2004). The previously reported *gyrA* mutations leading to Val149Ile, Asn203Ser, Ala206Val and Ala206Thr amino acid substitutions (Ge *et al.* 2003) were not found in our study. The concomitant occurrence of the majority of these substitutions with the Thr86Ile GyrA substitution and the fact that these mutations lie outside of the described *Campylobacter gyrA* QRDR (Wang *et al.* 1993) makes it difficult to draw conclusions as to their contribution to quinolone resistance in *Campylobacter*. In

addition, the mutation leading to the Pro104Ser GyrA substitution, although rare, is always accompanied by the Thr86Ile substitution in quinolone resistant human *Campylobacter* isolates (Zirnstein *et al.* 1999; Hakanen *et al.* 2002; Ge *et al.* 2003; Piddock *et al.* 2003; Beckmann *et al.* 2004). Similarly, the Ser22Gly GyrA substitution, observed in isolates from chickens and turkeys (Ge, personal communication), and detected in *C. jejuni* chicken isolates from this study, is also found with the Thr86Ile substitution in quinolone resistant strains. Ser22 *gyrA* mutations have also been observed in CIP-sensitive *Campylobacter* isolates (Dionisi *et al.* 2004). This suggests that the *gyrA* mutations leading to Ser22Gly GyrA and Pro104Ser substitutions may not contribute to quinolone resistance, but instead may be indicative of *gyrA* alleles found in poultry and human *Campylobacter* isolates, respectively. Larger numbers of isolates having these mutations are needed to confirm this hypothesis.

The relationship between CmeB expression and quinolone resistance has not been fully determined. Luo *et al.* (2003) reported that the expression levels of CmeB and CmeC from CIP-resistant and CIP-sensitive *Campylobacter* chicken isolates were similar. In addition, Pumbwe *et al.* (2004) found that only 9 of 32 multiple-antibiotic resistant *C. jejuni* isolates had increased CmeB expression levels. The role of other quinolone resistance mechanisms in *Campylobacter* has been studied extensively by Pumbwe and Piddock, who have suggested that efflux pumps other than CmeABC, which can be found in the published *C. jejuni* genome (Parkhill *et al.* 2000), may play a role in quinolone resistance (Pumbwe and Piddock 2002; Pumbwe *et al.* 2004). Most recently, Pumbwe *et al.* (2005) reported new evidence for multiple-antibiotic resistance in *C. jejuni* not mediated by the efflux pumps CmeABC or the newly identified CmeDEF. Hence, the involvement of efflux pumps in conferring quinolone and fluoroquinolone resistance in *Campylobacter* appears to be more complex than previous studies have suggested.

Although it is well established that discrete point mutations in *gyrA* can confer resistance to both CIP and NAL, or to NAL alone, eight isolates were identified in the present study with MICs $<4\ \mu g\ ml^{-1}$ for CIP and $\geq 32\ \mu g\ ml^{-1}$ for NAL, but no detectable *gyrA* QRDR mutations. Further investigation is clearly required to fully define the mechanisms involved in CIP and NAL resistance in *Campylobacter*.

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References

Bachoual, R., Ouabdesselam, S., Mory, F., Lascols, C., Soussy, C.J. and Tankovic, J. (2001) Single or double mutational alterations of *gyrA* associated with fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli*. *Microb Drug Resist* **7**, 257–261.

Beckmann, L., Muller, M., Luber, P., Schrader, C., Bartelt, E. and Klein, G. (2004) Analysis of *gyrA* mutations in quinolone-resistant and -susceptible *Campylobacter jejuni* isolates from retail poultry and human clinical isolates by non-radioactive single-strand conformation polymorphism analysis and DNA sequencing. *J Appl Microbiol* **96**, 1040–1047.

Chu, Y.W., Chu, M.Y., Luey, K.Y., Ngan, Y.W., Tsang, K.L. and Kam, K.M. (2004) Genetic relatedness and quinolone resistance of *Campylobacter jejuni* strains isolated in 2002 in Hong Kong. *J Clin Microbiol* **42**, 3321–3323.

Chuma, T., Maeda, T., Niwa, H. and Okamoto, K. (2004) Acquisition of quinolone resistance and point mutation of the *gyrA* gene in *Campylobacter jejuni* isolated from broilers and in vitro-induced resistant strains. *J Vet Med Sci* **66**, 155–160.

Dionisi, A.M., Luzzi, I. and Carattoli, A. (2004) Identification of ciprofloxacin-resistant *Campylobacter jejuni* and analysis of the *gyrA* gene by the LightCycler mutation assay. *Mol Cell Probes* **18**, 255–261.

Englen, M.D. and Fedorka-Cray, P.J. (2002) Evaluation of a commercial diagnostic PCR for the identification of *Campylobacter jejuni* and *Campylobacter coli*. *Lett Appl Microbiol* **35**, 353–356.

Friedman, C.R., Neimann, J., Wegner, H.C. and Tauxe, R.V. (2000) Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In *Campylobacter*, 2nd edn. ed. Nachamkin, I. and Blaser, M.J. pp. 121–138. Washington, DC: American Society for Microbiology.

Ge, B., White, D.G., McDermott, P.F., Girard, W., Zhao, S., Hubert, S. and Meng, J. (2003) Antimicrobial-resistant *Campylobacter* species from retail raw meats. *Appl Environ Microbiol* **69**, 3005–3007.

Hakanen, A., Jalava, J., Kotilainen, P., Jousimies-Somer, H., Siitonen, A. and Huovinen, P. (2002) *gyrA* polymorphism in *Campylobacter jejuni*: detection of *gyrA* mutations in 162 *C. jejuni* isolates by single-strand conformation polymorphism and DNA sequencing. *Antimicrob Agents Chemother* **46**, 2644–2647.

Hirose, K., Hashimoto, A., Tamura, K., Kawamura, Y., Ezaki, T., Sagara, H. and Watanabe, H. (2002) DNA sequence analysis of DNA gyrase and DNA topoisomerase IV quinolone resistance-determining regions of *Salmonella enterica* serovar Typhi and serovar Paratyphi A. *Antimicrob Agents Chemother* **46**, 3249–3252.

Ling, J.M., Chan, E.W., Lam, A.W. and Cheng, A.F. (2003) Mutations in topoisomerase genes of fluoroquinolone-resistant salmonellae in Hong Kong. *Antimicrob Agents Chemother* **47**, 3567–3573.

Luber, P., Bartelt, E., Genschow, E., Wagner, J. and Hahn, H. (2003) Comparison of broth microdilution, E Test, and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni* and *Campylobacter coli*. *J Clin Microbiol* **41**, 1062–1068.

Luo, N., Sahin, O., Lin, J., Michel, L.O. and Zhang, Q. (2003) In vivo selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with *gyrA* mutations and the function of the CmeABC efflux pump. *Antimicrob Agents Chemother* **47**, 390–394.

Maxwell, A. (1992) The molecular basis of quinolone action. *J Antimicrob Chemother* **30**, 409–414.

McIver, C., Hogan, T., White, P. and Tapsall, J. (2004) Patterns of quinolone susceptibility in *Campylobacter jejuni* associated with different *gyrA* mutations. *Pathology* **36**, 166–169.

Niwa, H., Chuma, T., Okamoto, K. and Itoh, K. (2003) Simultaneous detection of mutations associated with resistance to macrolides and quinolones in *Campylobacter jejuni* and *C. coli* using a PCR-line probe assay. *Int J Antimicrob Agents* **22**, 374–379.

Oncul, O., Zarakolu, P., Oncul, O. and Gur, D. (2003) Antimicrobial susceptibility testing of *Campylobacter jejuni*: a comparison between Etest and agar dilution method. *Diagn Microbiol Infect Dis* **45**, 69–71.

Padungtod, P., Kaneene, J.B., Wilson, D.L., Bell, J. and Linz, J.E. (2003) Determination of ciprofloxacin and nalidixic acid resistance in *Campylobacter jejuni* with a fluorogenic polymerase chain reaction assay. *J Food Prot* **66**, 319–323.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M. *et al.* (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**, 665–668.

Payot, S., Dridi, S., Laroche, M., Federighi, M. and Magras, C. (2004) Prevalence and antimicrobial resistance of *Campylobacter coli* isolated from fattening pigs in France. *Vet Microbiol* **101**, 91–99.

Piddock, L.J., Ricci, V., Pumbwe, L., Everett, M.J. and Griggs, D.J. (2003) Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. *J Antimicrob Chemother* **51**, 19–26.

Pumbwe, L. and Piddock, L.J. (2002) Identification and molecular characterisation of CmeB, a *Campylobacter jejuni* multidrug efflux pump. *FEMS Microbiol Lett* **206**, 185–189.

Pumbwe, L., Randall, L.P., Woodward, M.J. and Piddock, L.J. (2004) Expression of the efflux pump genes *cmeB*, *cmeF* and the porin gene *porA* in multiple-antibiotic-resistant *Campylobacter jejuni*. *J Antimicrob Chemother* **54**, 341–347.

Pumbwe, L., Randall, L.P., Woodward, M.J. and Piddock, L.J. (2005) Evidence for multiple-antibiotic resistance in *Campylobacter jejuni* not mediated by CmeB or CmeF. *Antimicrob Agents Chemother* **49**, 1289–1293.

Skirrow, B. and Blaser, M.J. (2000) Clinical aspects of *Campylobacter* infection. In *Campylobacter*, 2nd edn. ed. Nachamkin, I. and Blaser, M.J. pp. 69–88. Washington, DC: American Society for Microbiology.

Stern, N.J., Wojton, B. and Kwiatek, K. (1992) A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni*. *J Food Prot* **55**, 514–517.

Wang, Y., Huang, W.M. and Taylor, D.E. (1993) Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. *Antimicrob Agents Chemother* **37**, 457–63.

Willmott, C.J. and Maxwell, A. (1993) A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. *Antimicrob Agents Chemother* **37**, 126–127.

Wilson, D.L., Abner, S.R., Newman, T.C., Mansfield, L.S. and Linz, J.E. (2000) Identification of ciprofloxacin-resistant *Campylobacter jejuni* by use of a fluorogenic PCR assay. *J Clin Microbiol* **38**, 3971–3978.

Zirnstein, G., Helsel, L., Li, Y., Swaminathan, B. and Besser, J. (2000) Characterization of *gyrA* mutations associated with fluoroquinolone resistance in *Campylobacter coli* by DNA sequence analysis and MAMA PCR. *FEMS Microbiol Lett* **190**, 1–7.

Zirnstein, G., Li, Y., Swaminathan, B. and Angulo, F. (1999) Ciprofloxacin resistance in *Campylobacter jejuni* isolates: detection of *gyrA* resistance mutations by mismatch amplification mutation assay PCR and DNA sequence analysis. *J Clin Microbiol* **37**, 3276–3280.